



## Three new species of *Candida* from apple cider: *C. anglica*, *C. cidri* and *C. pomicola*

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### Abstract

Three new anamorphic ascomycetous yeasts are described: *Candida anglica* (type strain NRRL Y-27079, CBS 4262), *Candida cidri* (type strain NRRL Y-27078, CBS 4241), and *Candida pomicola* (type strain NRRL Y-27083, CBS 4242). These three species were isolated from cider produced in the United Kingdom, and their identification was determined from unique nucleotide sequences in the species-specific D1/D2 domain of large subunit (26S) ribosomal DNA. Phylogenetic analysis of D1/D2 sequences placed *C. anglica* near *Candida fragi*, *C. cidri* near *Pichia capsulata*, and *C. pomicola* in the *Pichia holstii* clade.

### Introduction

Yeasts are often abundant in apple juice and cider, and these populations show a remarkable taxonomic diversity that includes numerous genera of both ascomycetous and basidiomycetous species (Beech & Davenport 1970, and references therein; Walker & Ayres 1970). F.W. Beech deposited a number of unidentified isolates from cider in the Yeast Collection of the Centraalbureau voor Schimmelcultures. Among these deposits were three asporogenous stains that failed to correspond with known species when compared from standard phenotypic tests (Kurtzman & Fell 1998; Meyer et al. 1998). This prompted examination of the strains from their nucleotide sequences in the species-specific D1/D2 domain of large subunit (26S) ribosomal DNA (rDNA) (Kurtzman & Robnett 1998). Sequence analysis showed that the three unidentified strains represent new ascomycetous yeasts, and they are described here as new members of the genus *Candida*.

### Materials and methods

#### *Organisms, morphological and physiological tests*

Strains of the new species studied are listed in Table 1, and all are maintained in the Agricultural Research Service Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, Illinois, USA, as well as in the Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands. The strains were received at CBS from F.W. Beech as unidentified isolates, and have been maintained as unresolved species of *Candida*. The composition of culture media used in this study, as well as protocols for fermentation and assimilation tests, are given by Yarrow (1998).

#### *rDNA sequencing and sequence analysis*

Methods for nuclear DNA isolation, amplification of 26S rDNA domain D1/D2 by polymerase chain reaction (PCR), and sequencing with the ABI Taq-DyeDeoxy Terminator Cycle sequencing kit/ABI Model 377 automated DNA sequencer (Applied Biosystems, Inc., Foster City, California) were previously described (Kurtzman & Robnett 1998).

Table 1. Strains of the new *Candida* species described

Species	Strain designation <sup>1</sup>		GenBank accession no. for 26S D1/D2 rDNA sequence	Source
	NRRL	CBS		
<i>C. anglica</i>	Y-27079	4262	AF245403	Cider, F.W. Beech, United Kingdom
<i>C. cidri</i>	Y-27078	4241	AF245402	As above
<i>C. pomicola</i>	Y-27083	4242	AF245400	As above

NRRL, Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, Illinois, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

Sequence data were visually aligned with QEdit 2.15 (SemWare, Marietta, Georgia), and phylogenetic relationships were calculated using the maximum parsimony program of PAUP\* 4.0 (Swofford 1998) with heuristic searches employing both simple and random sequence additions. Relationships were further analyzed by the neighbor-joining program of PAUP\* 4.0 using the Kimura 2-parameter distance measure. *Schizosaccharomyces pombe* was the designated outgroup in all analyses, which initially included all currently recognized ascomycetous yeasts along with reference euascomycetes, 'archiascomycetes' and basidiomycetes. Confidence limits for phylogenetic trees were estimated from bootstrap analyses (1000 replications). The nucleotide sequences of the new species have been deposited with GenBank under the accession numbers given in Table 1; sequences of other species used in analyses were previously deposited in GenBank (Kurtzman & Robnett 1998).

## Results

Ascomycetous yeasts that differ from one another by greater than 1% substitutions in the variable 600-nucleotide D1/D2 domain of large subunit rDNA have been shown to represent different species (Kurtzman & Robnett 1995, 1998), and at present, there are no known exceptions to this observation. The three new species proposed in this report differ from each other and from all currently recognized ascomycetous yeasts by greater than 1% substitutions and are expected to represent independent, genetically isolated lineages. The relationships of these three taxa with known species were determined from phylogenetic analysis of D1/D2 domain sequences (Figures 1 and 2). Ascosporeulation has not been observed in these species and they

are assigned to the genus *Candida* because vegetative reproduction is by multilateral budding.

*Candida anglica* Kurtzman, Robnett & Yarrow, sp. nov.

In agar multi post dies 3 ad 25 °C, cellulae vegetativae globosae (4.0–7.5 µm diam.) ad ellipsoideae (2.1–5.5 × 3.5–18.0 µm), singulae vel binae. In agar morphologico post dies 7 ad 25 °C, incrementum fuscum pallidum, hebes vel nitens et butyrosom. Centrum colonia convexum, margine glabro vel undulato. Pseudohyphae adsunt negue; hyphae verae non fiunt. Ascosporae absentes.

Glucosum, galactosum (variabile) et trehalosum fermentantur. Sucrosom, maltosom, lactosom, et raffiniosom non fermentantur. Assimilantur glucosum, galactosom, L-sorbosom, trehalosom, ethanolum, glycerolum, ribitolum, xylitolum, D-mannitolum, D-glucitolum, D-gluconatum, D-glucono-1,5-lactonum, 2-keto-D-gluconatum, acidum succinicum, acidum citricum, cadaverinum, ethylaminum, et L-lysinum. Non assimilantur sucrosom, maltosom, cellobiosom, lactosom, melibiosom, raffiniosom, melezitiosom, inulinum, amyllum solubile, D-xylosom, L-arabiosom, D-arabiosom, D-ribosom, L-rhamnosom, D-glucosaminum, methanolum, erythritolum, L-arabinitolum, galactitolum, methyl-α-D-glucosidum, salicinum, arbutinum, D-glucuronatum, D-galacturonatum, hemisaccharatum, D-galactonatum, propanediolum, butanediolum, acidum quinicum, DL-acidum lacticum, inositolum, potassii nitratum, potassii nitritum, creatinum, creatininum, D-glucosaminum (nitrogenium), imidazolium, et D-tryptophanum. Vitamina externa crescentiae necessaria sunt. Temperatura 37 °C non crescit.

Typus. Holotypus NRRL Y-27079 (CBS 4262) lyophilus. Cultura isolata vino malis confecto in

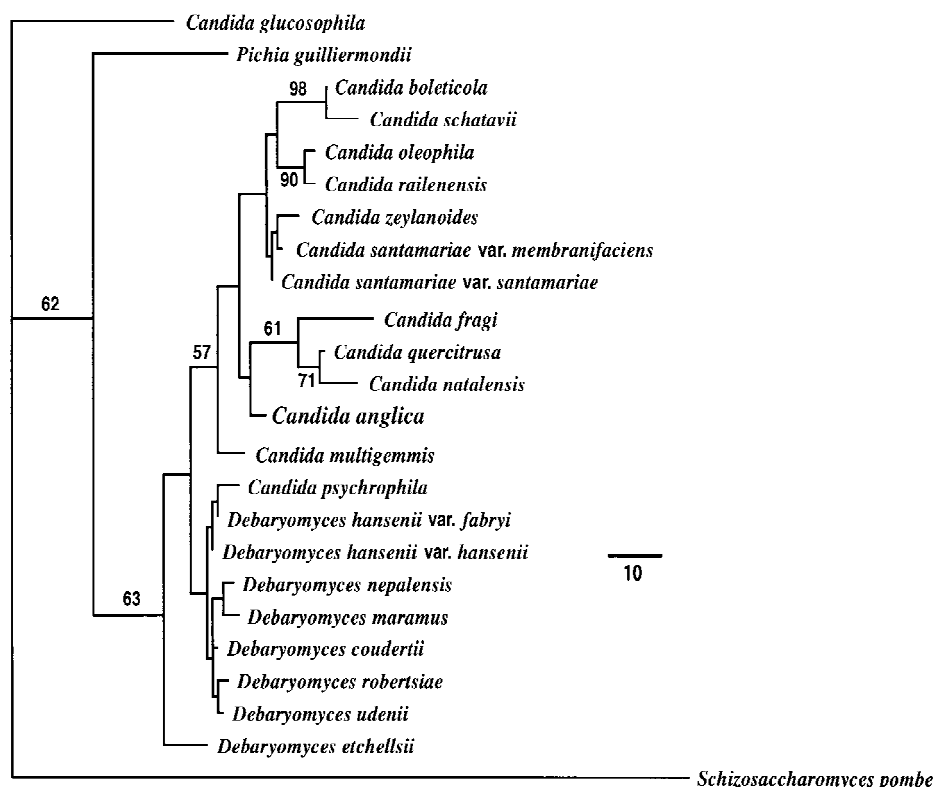


Figure 1. Phylogenetic placement of *Candida anglica* among related ascomycetous yeasts as represented by 1 of 28 most parsimonious trees derived from maximum parsimony analysis of domain D1/D2 26S rDNA. Tree length = 317 steps, consistency index (CI) = 0.726, retention index (RI) = 0.660, rescaled consistency index (RC) = 0.479, homoplasy index (HI) = 0.274, number of parsimony-informative characters = 68. Branch lengths are proportional to nucleotide differences (Bar = 10 nucleotides), and the numbers given at nodes are the percentage of frequencies with which a given branch appeared in 1000 bootstrap replications. Frequencies under 50% are not given. *Schizosaccharomyces pombe* was designated as the outgroup species for phylogenetic analysis.

Anglica. Deposita in Collectione Culturarum ARS (NRRL), Peoria, Illinois, USA.

#### Description of *Candida anglica*

**Growth on 5% malt extract agar.** After 3 days at 25 °C, the cells are spherical (4.0–7.5 µm diam.) to ellipsoidal (2.1–5.5 × 3.5–18.0 µm), and occur singly and in pairs (Figure 3). Budding is multilateral. Growth is tannish-white, dull, butyrous and with a thin border of pseudomycelium.

**Dalmau plate culture on morphology agar.** After 7 days at 25 °C, moderately branched pseudohyphae with blastoconidia were formed under the coverglass (Figure 4). True hyphae were not observed. Aerobic growth is tannish-white, semi-glistening, coarsely striated and butyrous. The colony margin is finely lobed.

**Examination for the presence of an ascosporic state.** Ascospores were not detected in the type strain of *C. anglica* when grown for up to 3 months on YM, 5% malt extract, McClary's acetate and RG agars incubated at 15° and 25 °C.

**Physiological tests.** Reactions on the fermentation, assimilation and other growth tests commonly used in yeast taxonomy are given in Table 2.

**Type.** NRRL Y-27079 (CBS 4262) is preserved as a lyophilized preparation in the Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois, USA. The strain was isolated from apple cider in England (Table 1).

**Etymology.** The species name *anglica* refers to England, the country from which the type strain was isolated.

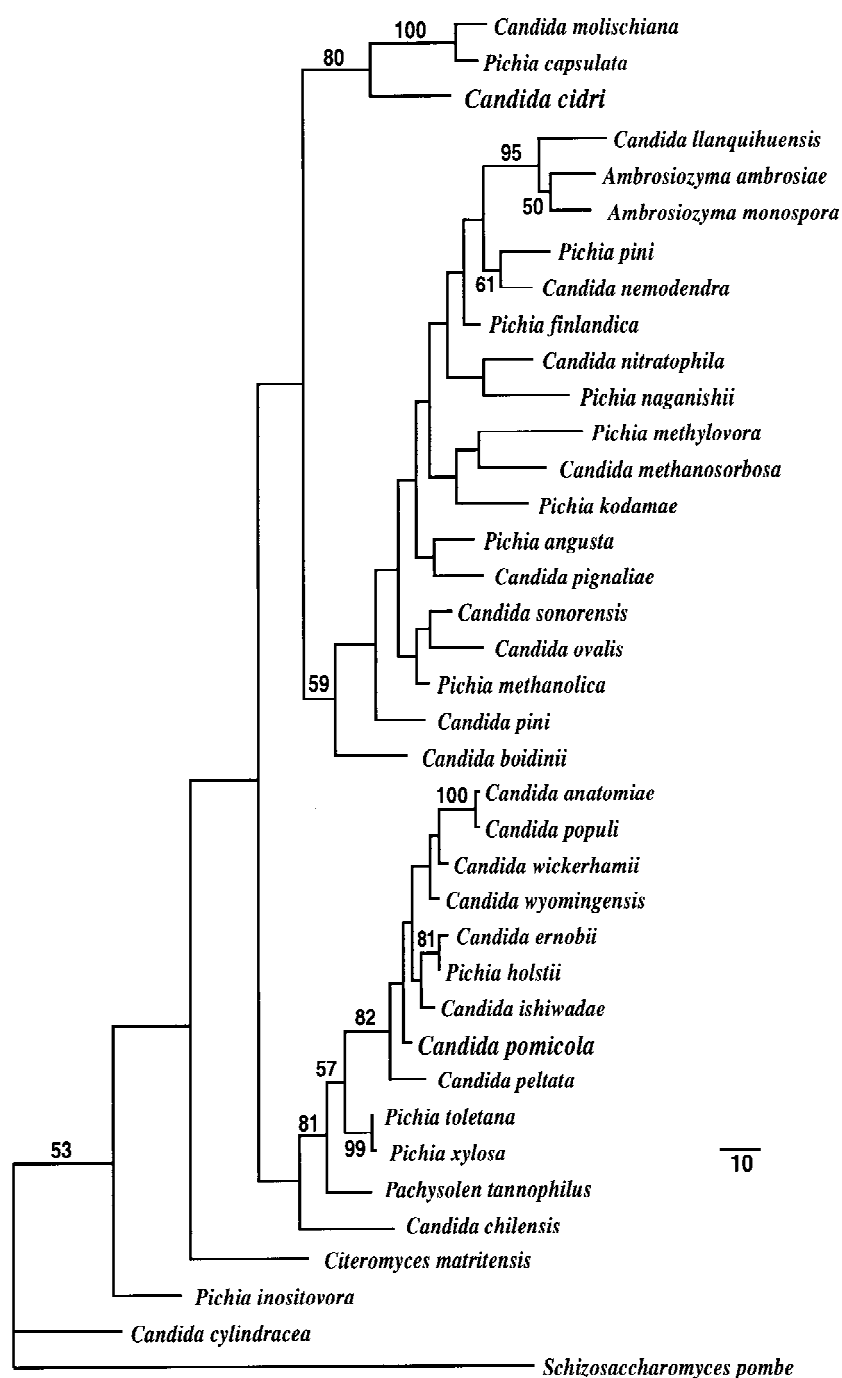
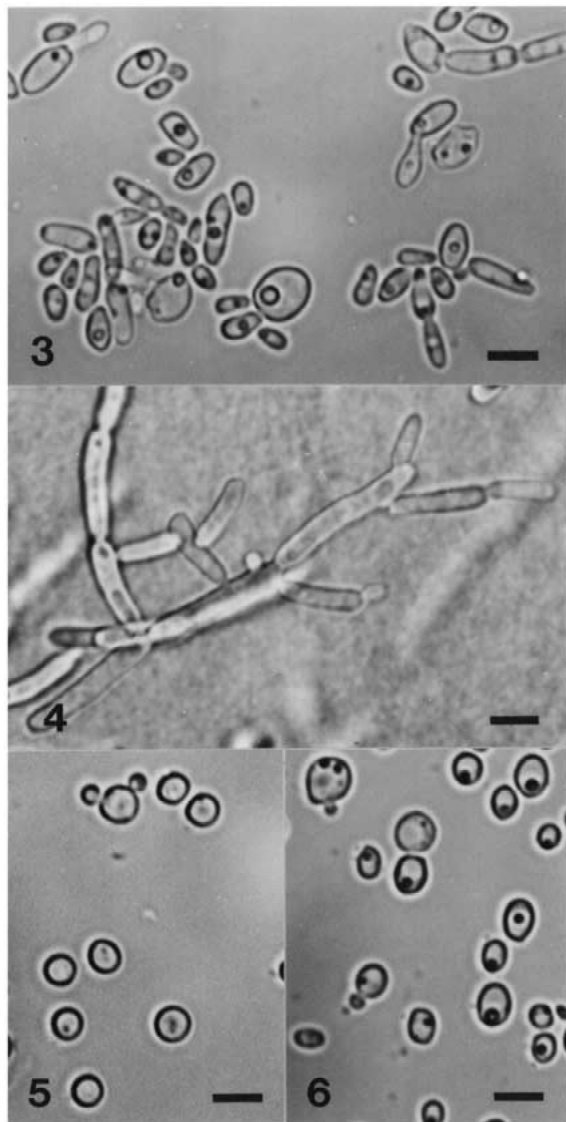


Table 2. Physiological characteristics of *Candida anglica*, *C. cidri* and *C. pomicola*

Physiological Test	Reaction of <sup>1</sup>			Physiological Test	Reaction of		
	<i>C. anglica</i>	<i>C. cidri</i>	<i>C. pomicola</i>		<i>C. anglica</i>	<i>C. cidri</i>	<i>C. pomicola</i>
Fementation:							
Glucose	+	+	+	Lactose	—	—	—
Galactose	v	—	—	Raffinose	—	—	—
Sucrose	—	—	—	Trehalose	+	v	v
Maltose	—	—	—				
Assimilation:							
Glucose	+	+	+	Ribitol	+	+	+
Galactose	+	—	+D	Xylitol	+D	+	+
L-Sorbose	+	+	v	L-Arabinitol	—	+	+, w
Sucrose	—	v	+	Galactitol	—	—	—
Maltose	—	+	+	D-Mannitol	+	+	+
Cellobiose	—	—	+	D-Glucitol	+	+	+
Trehalose	+	+	+	Methyl- $\alpha$ -D-glucoside	—	v	+D
Lactose	—	—	—	Salicin	—	—	+
Melibiose	—	—	—	Arbutin	—	—	+
Raffinose	—	—	—	D-Gluconate	+	—	+
Melezitose	—	+D	+	D-Glucono-1,5-lactone	+	+, w	+
Inulin	—	—	—	2-Keto-D-gluconate	+	—	—
Soluble Starch	—	+	+	D-Glucuronate	—	—	—
D-Xylose	—	+	+	D-Galacturonate	—	+	—
L-Arabinose	—	+D	+	Hemi-Saccharate	—	—	—
D-Arabinose	—	+	+, w	D-Galactonate	—	—	—
D-Ribose	—	+	+	Propanediol	—	+D	+D
L-Rhamnose	—	+	+	Butanediol	—	—	—
D-Glucosamine	—	+	+D	Quinic acid	—	—	—
Methanol	—	+	—	DL-Lactate	—	+	—
Ethanol	+	+	+	Succinate	+	+	+
Glycerol	+	+	+	Citrate	+	+	+
Erythritol	—	+	+, w	Inositol	—	—	—
Nitrogen sources:							
Nitrate	—	+	+	Ethylamine	+	+	+
Nitrite	—	+	+	D-Glucosamine	—	+	+
Cadaverine	+	+	+	Imidazole	—	—	—
Creatine	—	—	—	L-Lysine	+	+	+
Creatinine	—	—	—	D-Tryptophan	—	—	—
Additional growth tests:							
Vitamin-free medium	—	—	—	Cycloheximide, 0.01%	—	+	+
NaCl 10%	+	+	+	Cycloheximide, 0.1%	—	+	+
NaCl 16%	—	—	—	Growth at 37 °C	—	—	—

<sup>1</sup>—, negative; +, positive; +D, positive but delayed growth; w, weak; v, variable, i.e., + or —. Note that the variable reactions listed, as well as those given as +, w, resulted because of different responses when the tests were repeated. Delayed reactions (+D) might potentially have been variable upon repeat.



Figures 3–6. *Candida anglica* NRRL Y-27079. 3. Budding cells, 2 days, YM agar, 25 °C. 4. Pseudohyphae, 7 days, Dalmau plate culture on yeast morphology agar, 25 °C. *Candida cidri* NRRL Y-27078. 5. Budding cells, 6 hours, RG agar, 25 °C. *Candida pomicola* NRRL Y-27083. 6. Budding cells, 7 hours, RG agar, 25 °C. Bars = 5 µm.

As shown in Figure 1, the nearest known neighbors of *Candida anglica* are *Candida fragi*, *C. quercitrusa* and *C. natalensis*. These species differ, respectively, from *C. anglica* by the following percent nucleotide substitutions in domain D1/D2: 5.7, 5.0 and 5.0%. *C. anglica* can be separated from these three species on assimilation tests by its lack of growth on sucrose.

*Candida cidri* Kurtzman, Robnett & Yarrow, sp. nov.

In agaro malti post dies 3 ad 25 °C, cellulae vegetativae globosae (2.3–6.0 µm diam.), singulae vel binae. In agaro morphologico post dies 7 ad 25 °C, incrementum fuscum pallidum, nitens et butyrosus vel mucosus. Centrum colonia convexum, margine undulato. Pseudohyphae et hyphae verae absentes. Ascospores absentes.

Glucosum et trehalosum (variabile) fermentantur. Galactosum, sucrosus, maltosus, lactosus, et raffinosis non fermentantur. Assimilantur glucosus, L-sorbosus, sucrosus (variabile), maltosus, trehalosus, melezitosis, amyllum solubile, D-xylosus, L-arabinosus, D-arabinosus, D-ribosus, L-rhamnosus, D-glucosaminus, methanolus, ethanolus, glycerolus, erythritolus, ribitolus, xylitolus, L-arabinitolus, D-mannitolus, D-glucitolus, methyl- $\alpha$ -D-glucosidus (variabile), D-glucono-1,5-lactonus, D-galacturonatus, propanediolus, DL-acidus lacticus, acidus succinicus, acidus citricus, potassii nitratus, potassii nitritus, cadaverinus, ethylaminus, D-glucosaminus (nitrogenium), et L-lysinus. Non assimilantur galactosus, cellobiosus, lactosus, melibiosus, raffinosis, inulinus, galactitolus, salicinus, arbutinus, D-gluconatus, 2-keto-D-gluconatus, D-glucuronatus, hemi-saccharatus, D-galactonatus, butanediolus, acidus quinicus, inositolus, creatinus, creatininus, imidazolus, et D-tryptophanus. Vitamina externa crescentiae necessaria sunt. Temperatura 37 °C non crescit.

Typus. Holotypus NRRL Y-27078 (CBS 4241) lyophilus. Cultura isolata vino malis confecto in Anglica. Deposita in Collectione Culturarum ARS (NRRL), Peoria, Illinois, USA.

#### Description of *Candida cidri*

**Growth on 5% malt extract agar.** After 3 days at 25 °C, the cells are spherical (2.3–6.0 µm diam.) and single or infrequently in pairs (Figure 5). Budding is multilateral. Growth is tannish-white, glistening and butyrous to nearly mucoid.

**Dalmau plate culture on morphology agar.** After 7 days at 25 °C, moderately branched rosettes of undifferentiated cells were formed under the coverglass, but pseudohyphae and true hyphae were not formed. Aerobic growth is tannish-white, smooth, glistening and butyrous. The colony margin is irregularly lobed.

*Examination for the presence of an ascospore state.* Ascospores were not detected in the type strain of *C. cidri* when grown for up to 3 months on YM, 5% malt extract, McClary's acetate and RG agars incubated at 15° and 25 °C.

*Physiological tests.* Reactions on the fermentation, assimilation and other growth tests commonly used in yeast taxonomy are given in Table 2.

*Type.* NRRL Y-27078 (CBS 4241) is preserved as a lyophilized preparation in the Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois, USA. The strain was isolated from apple cider in England (Table 1).

*Etymology.* The species name *cidri* refers to cider, the substrate from which the type strain was isolated.

*Candida cidri* is only the third known member of the small clade that includes *Pichia capsulata* and *Candida molischiana* (Figure 2). *Candida cidri* differs from these two species by, respectively, 8.8% and 9.2% nucleotide substitutions in the D1/D2 domain. All three species are characterized by production of copious amounts of extracellular polysaccharides (Kurtzman 1998). On assimilation tests, *C. cidri* differs from *P. capsulata* and *C. molischiana* by its inability to grow on cellobiose and salicin as sole sources of carbon.

*Candida pomicola* Kurtzman, Robnett & Yarrow, sp. nov.

In agar malti post dies 3 ad 25 °C, cellulae vegetativae globosae (2.3–5.0 µm diam.) ad ellipsoideae (1.8–4.3 × 2.1–6.0 µm), singulae vel binae. In agar morphologico post dies 7 ad 25 °C, incrementum fuscum pallidum, nitens et butyrosus. Centrum colonia sublatum, margine glabro vel undulato. Pseudohyphae et hyphae verae adsunt. Ascosporae absentes.

Glucosum et trehalosum (variabile) fermentantur. Galactosum, sucrosus, maltosus, lactosus, et raffinosis non fermentantur. Assimilantur glucosus, galactosus, L-sorbosus (variabile), sucrosus, maltosus, cellobiosus, trehalosus, melezitosis, amyllum solubile, D-xylosus, L-arabiosus, D-arabiosus, D-ribosus, L-rhamnosus, D-glucosaminus, ethanolum, glycerolum, erythritolum, ribitololum, xylitololum, L-arabinitolum, D-mannitololum, D-glucitololum, methyl-α-D-glucosidus, salicinum, arbutinum, D-gluconatum, D-glucono-1,5-lactonum, pro-

panediolum, acidum succinicum, acidum citricum, potassii nitratum, potassii nitritum, cadaverinum, ethylaminum, D-glucosaminum (nitrogenium), et L-lysinum. Non assimilantur lactosus, melibiosus, raffinosis, inulinum, methanolum, galactitololum, 2-keto-D-gluconatum, D-glucuronatum, D-galacturonatum, hemi-saccharatum, D-galactonatum, butanediolum, acidum quinicum, DL-acidum lacticum, inositololum, creatinum, creatininum, imidazololum, et D-tryptophanum. Vitamina externa crescentiae necessariae sunt. Temperatura 37 °C non crescit.

*Typus.* Holotypus NRRL Y-27083 (CBS 4242) lyophilus. Cultura isolata vino malis confecto in Anglica. Deposita in Collectione Culturarum ARS (NRRL), Peoria, Illinois, USA.

#### *Description of Candida pomicola*

*Growth on 5% malt extract agar.* After 3 days at 25 °C, the cells are spherical (2.3–5.0 µm diam.) to ellipsoidal (1.8–4.3 × 2.1–6.0 µm), and are single or occasionally in pairs (Figure 6). Budding is multilateral. Growth is tannish-white, glistening and butyrous. Growth of *C. cidri* and *C. pomicola* on common culture media results in a profusion of extracellular polysaccharide that blurs photographic images of the cells. Consequently, the cells shown in Figures 5 and 6 are from low nutrient restricted growth (RG) agar.

*Dalmau plate culture on morphology agar.* After 7 days at 25 °C, moderately branched rosettes of undifferentiated cells are seen under the coverglass, but pseudohyphae and true hyphae are not formed. Aerobic growth is tannish-white, smooth, glistening and butyrous. The colony margin has small, irregular lobes.

*Examination for the presence of an ascospore state.* Ascospores were not detected in the type strain of *C. pomicola* when grown for up to 3 months on YM, 5% malt extract, McClary's acetate and RG agars incubated at 15° and 25 °C.

*Physiological tests.* Reactions on the fermentation, assimilation and other growth tests commonly used in yeast taxonomy are given in Table 2.

*Type.* NRRL Y-27083 (CBS 4242) is preserved as a lyophilized preparation in the Agricultural Research Service Culture Collection (NRRL), Peoria, Illinois, USA. The strain was isolated from apple cider in England (Table 1).

**Etymology.** The species name *pomicola* refers to apples to denote the isolation of the type strain from apple cider.

On the basis of phylogenetic analysis of domain D1/D2 sequences, *Candida pomicola* is a member of the clade that includes *Pichia holstii* and *Pachysolen tannophilus* (Figure 2). Some members of this clade produce abundant extracellular polysaccharides resulting in mucoid growth like that of *P. holstii* and *P. tannophilus*, whereas other species do not demonstrate this property and colony growth is butyrous. *Candida pomicola* is most closely related to the following four species which are listed along with extent of nucleotide divergence from *C. pomicola*: *Candida wyomingensis* (1.3%), *C. ernobii* (1.7%), *C. ishiwadae* (1.3%), and *Pichia holstii* (1.7%). On standard fermentation and assimilation tests, *C. pomicola* differs from *C. ishiwadae* by failure to ferment maltose and from *C. wyomingensis* and *C. ernobii* by their inability to assimilate melezitose. Differentiation of *C. pomicola* and *P. holstii* can be made microscopically from the presence of true hyphae in cultures of *P. holstii*.

## Discussion

The extensive studies of Beech and many others, as reviewed by Beech & Davenport (1970), have demonstrated that apple juice and cider are luxuriant growth media for many yeasts, and the sources for a majority of these species appear to be from infested blossoms, leaves and fruits. Walker & Ayres (1970) have further documented yeast spoilage in a large variety of foods and beverages. Effective control of spoilage is dependent on accurate identification of the spoilage organisms and detection of their sources in the environment.

The application of molecular methods to yeast taxonomy has markedly increased the accuracy and rapidity of strain identification. Methods are still being developed that rely on various gene sequences and different detection methodologies. One of the gene sequences of particular interest is the ca. 600-nucleotide D1/D2 domain of large subunit rDNA, which has been sequenced for all currently accepted ascomycetous and basidiomycetous yeasts (Kurtzman & Robnett 1998; Fell et al. 2000). This easily sequenced region is sufficiently variable to allow detection of most species. Exceptions have been noted in which some closely related species have the same D1/D2 sequence (Kurtzman & Robnett 1998; Fell et al. 2000). However, for

ascomycetous yeasts, there are at present no known exceptions to the observation that strains that differ by more than 1% substitutions represent separate species. As a result, comparison of sequence divergence in the D1/D2 domain provides a rapid determination for most species and can serve as the basis for development of molecular diagnostic technologies needed in the field of food safety.

## Acknowledgements

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The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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